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Association of Reduced Folate Carrier (RFC) Gene Polymorphism with Colorectal Cancer Susceptibility in Kashmir.

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Abstract

Folate, important cofactorin one carbon moiety transfer, has been a factor that may modulate the development of colorectal cancer through aberrant DNA methylation and altered nucleotide synthesis and repair. Major folate transport across cell membrane is mediated by reduced folate carrier-1 (RFC1) that also preferably transports chemotherapeutic agents. Variants within the RFC 1 by influencing folate uptake may lead to colorectal cancer susceptibility. Our study is the first to investigate prospectively the RFC gene polymorphism in colorectal cancer in Kashmiri population. A total of 620 subjects (300 colorectal cancer patients and 320 normal subjects) were analyzed by PCR-RFLP technique for RFC gene polymorphism in exon 2 at position 80. We observed 1.27 fold increased risk for AA homozygous variant (OR= 1.27; 95% CI, 0.8678 - 1.875) and 1.19 fold increased risk for GA heterozygous genotypes (OR= 1.19; 95% CI, 0.8080 - 1.760) respectively to CRC susceptibility. However the statistically significant results for smoking and tumor location characteristics were stratified with RFC1 polymorphism, which suggests a possible effect of smoking and cancer location in the etiology of CRC in Kashmir.

Keywords:colorectal cancer; cancer etiology; Folate metabolizing genes; RFC gene polymorphism; RFC

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1. Introduction

Folate (an essential micronutrient in humans) has important biological function of providing single carbon units (methyl groups) required for intracellular methylation reactions and de novo DNA and amino acid metabolism, through folate metabolism pathway. Such importance suggests that folate deficiency [1, 2] and polymorphic variants in genes metabolizing it may modulate risk of colorectal cancer through aberrant DNA methylation and altered nucleotide synthesis and repair.

From various contradictory studies, dual role of folate has been hypothesized [3, 4, 5], which is, that folate deficiency in normal colorectal tissues may promote carcinogenesis, while as deficiency of folate could pose an inhibitory effect on the progression of established neoplasms [6, 3]. As folates (hydrophilic anionic molecules) cannot cross cell membranes by diffusion alone so require membrane transport systems for cellular uptake [7]. RFC (reduced folate carrier) is the major transport system in mammalian cells for folate, actively transports folate compounds into cells from the plasma and plays a role in maintaining intracellular concentrations of folate [7]. This saturable anion dependent membrane carrier appears to function optimally at physiological pH [8]. It has a greater affinity for reduced folates like 5-methyl THF and 5-formyl THF (Km = 1 - 5 μM) and is ubiquitously expressed in normal epithelial cells and cancer of epithelial origin [9, 10].

In addition to its role in folate uptake, RFC1 has a critical role in homeostasis of folate in mammalian cells, where its down regulation occurs as a response to folate deficiency [11]. Although a number of studies have examined the effect of single point mutations on RFC protein stability and function [12, 13], little is known about the mechanism of folate translocation into the cell. The RFC system also mediates internalization of antifolates including methotrexate (MTX) used to treat cancers, into cells [14,15]. Membrane transport of anti-folates by RFC is essential to the chemotherapeutic activity of such drugs, providing optimal intracellular drug concentrations to enable inhibition of target enzymes [16]. Therefore genetic variations in the gene may affect the transport of these drugs too [17]. Indeed, decreased RFC function results in resistance to anti-folate agents [15].This anion exchanging concentrative process is opposed by independent exit pumps that are directly coupled tometabolism of energy. These balanced processes inturngoverns the free intracellular folate level.

A common non-synonymous SNP Arg27His (80G>A; rs1051266 found across all ethnic groups is the most extensively studied variant of the RFC1 gene, in exon 2 that results in substitution of a histidine for an arginine at residue 27 in the protein sequence [18]. This variant has been widely studied for its function in transport uptake as well as its association with risk to cancers and drug response and toxicity. It was also suggested that the location of the RFC 80 G>A polymorphism in a region important to substrate binding might affect folate transport [19]. Alterations in folate membrane transport by RFC may be further compounded by gene polymorphisms that result in changes in the catalytic activities of folate-dependent interconverting and biosynthetic enzymes such as 5,10-methylene tetra hydro folate reductase (MTHFR) that impact the intracellular distribution of individual reduced folate forms [20]. The genotype frequencies for the RFC show marked geographic and ethnic variation, and this can in turn modulate the cancer risk in different geographical regions depending on the folate intake [21].The associations between RFC gene 80G>A polymorphism and CRC risk has been investigated in several previous studies [21-29].

Kashmir with distinct cultural, socio-economic and lifestyle trends, has been reported as a high incidence area of GIT cancers [30, 31]. In Kashmir valley, CRC is the third most common GIT cancer [32, 33]. Most of the work done in Kashmir has primarily focused on upper GIT cancers [30, 34]. and inspite of a high incidence CRC [32, 33], this area has

been undermined. In light of this, we designed a prospective, case-control study in a homogeneous sample of 620 individuals (300 cases and 320 controls). The aim was to investigate -i) the polymorphic spectrum of crucial folate transporting RFC gene in the ethnic population ii) to associate the possible risk, if any, of these polymorphisms withCRC susceptibility and iii) to associate various CRC risk factors in these patients with the disease susceptibility.

2. Materials and Method

2.1. Study population

The study was carried out in the Department of Biochemistry, University of Kashmir, in collaboration with the Department of General Surgery, at Sher-i- Kashmir Institute of Medical Science (SKIMS) Hospital, Srinagar, from where patients were recruited over a period of 4 years, following proper approval. The diagnosis of CRC was based on the standard colonoscopic/sigmoidoscopic methods (flexible type) and histopathological criteria. Controls (matched to cases by gender, age)were taken from healthy individuals of Kashmir valley from same division showing no clinical evidence suggestive of presence of CRC or any other bowel related diseases that might affect the study. Informed consent was obtained from each subject and personal data from each participant regarding demographic characteristics, such as sex, age, and related risk factors including smoking were collected via questionnaire. 3ml of venous blood sample was obtained from each subject and then immediately shifted and stored at -80°C until DNA was extracted. Present study included a total of 300 colonoscopically and histologically confirmed CRC patients as cases and 320 healthy malignancy free individuals as controls. Commercially available, genomic DNA purification kit (Genetix Biotech Asia, Pvt Ltd.) was used to extract the DNA from the blood samples of Subjects both cases and controls.

2.2. Genotyping

Desired region of the genome was amplified using the specific primer set consisting of RFC1- Forward: 5'-AGTGTCACCTTCGTCCC-3' and Reverse: 5'- TCCCGCGCGTGAAGTTCTTG-3' sequences that resulted in the amplification of 230 bp fragment. PCR was carried out in a final volume of 25µl reaction mixture that consisted of 50-75 ng of genomic DNA template, 0.5µM of each primer(Genescript), 12.5µl of Maxima Hot start master mix (Fermentas/ Thermoscientific) (along with nuclease free water added accordingly). The conditions were selected after extensively standardizing all the parameters. PCR technique applied to amplify the polymorphic site of RFC1, consisted of an initial denaturation for 2 min, followed by 35 cycles each of 30s at 94^{o}C, 30s annealing phase at 52^{o}C and 45s extension phase at 72^{o}C and ultimately a 7 min final extension at 72^{o}C was used to complete the reaction. The A-to-G transition at position 80 creates a HhaI (GCG/C) restriction site, amplicons were treated with HhaI (Fermentas) (1U at 37^{o}C) enzyme and kept for 3 hours which resulted in three fragments of 125, 68, and 37 bp, in the presence of the 80G allele, while the 80A allele produced two fragments of 162 and 68 bp. These were then electrophorised on ethidium bromide treated 3% agarose gel (Sigma Aldrich).

3. Statistical Analysis

GraphPad Prism version 6.0, softwarewas used to carry out the statistical analysis on the data.Results were expressed as means ± Standard deviation. Values of $p<0.05$ were considered to indicate statistical significance. Expected genotype frequencies were calculated from allele frequencies under the assumption of Hardy-Weinberg equilibrium. The differences in allele frequencies between cases and control were determined using chi-square test .The interaction

between RCF1 genotypes was evaluated by calculating the odds ratios (OR) for mutant genotypes, as compared to wild types. 95% confidence intervals (95% CI) were calculated to estimate the risk of the different genotypes.

4. Results

Present study included a total of 165 male and 135 female cases (M/F ratio-1.22); and 167 male and 153 female (M/F ratio- 1.09) control subjects. Table 1 presents selective general characteristics of cases and controls that were included in the present study. Mean age of cases was found to be 54.40 ± 13.45 (SD) while as in controls it was 53.80 ± 13.25 (SD). Since no significant differences were observed between cases and controls with respect to various characteristics ($p>0.05$), it suggested that frequency matching was adequate.

Table 1. General Characteristics of Study Population (cases and controls)

Variables	Cases (n=300)		Controls (n=320)		p value
	n	(%)	n	(%)	
Age					
≤ 50 yrs	122	(40.6%)	134	(41.87%)	**0.76**
> 50 yrs	178	(59.4%)	186	(58.13%)	
Gender:					
Male	165	(55%)	167	(52.19 %)	***0.48***
Female	135	(45%)	153	(47.81 %)	
Dwelling:					
Urban	81	(27%)	83	(25.93%)	**0.76**
Rural	219	(73%)	237	(74.07%)	
Smoking:					
Smokers	153	(51.0%)	175	(54.68%)	**0.35**
Non smokers	147	(49.0%)	145	(45.32%)	

n= No. of individuals; *P using $\chi 2$ Test (P<0.05, Data statistically significant)

Out of 300 cases, 81 (27%) belonged to urban region,219 (73%) to rural ones; 122 (40.6%) were either below or of 50 years of age, 178(59.4%) were above 50 years of age and 153 (51%) carried out some type of smoking (Hukka or Cigarette either singly or in combination) or used to snuff. 157 (52.33%) patients carried the malignancy in colonic region and 143 (47.67%) had it in rectal region. It was observed that cases had higher percentage of subjects from rural areas, above 50 aged subjects, males and smokers as compared to urban dwellers, below 50 years aged subjects, females and non-smokers respectively.

The status of RCF1 G80A polymorphism in all subjects was addressed by PCR amplification of genomic DNA as described in Materialsand Methods section, followed by restriction digestion with an appropriate endonuclease. The

results of the amplification are shown in the figure 2. Figure 3 shows the RFLP digestion profile of subjects under study for RFC homozygous and heterozygous variants. Table 2 and Table 3distributethe calculated allelic and genotypic frequencies of RFC1 gene in cases vs. controls respectively.

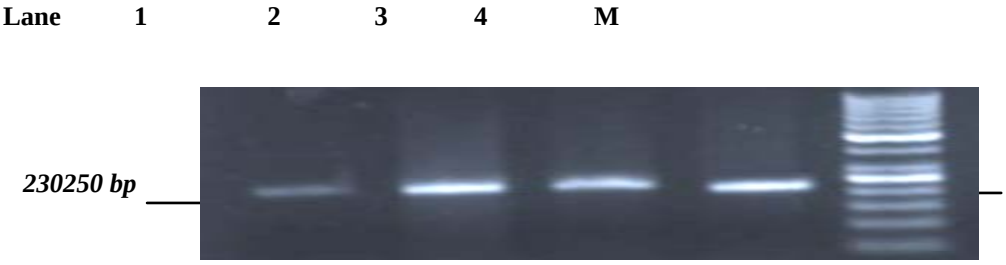

Figure 2: representative gel picture showing the PCR amplification products for RFC gene. Lane no. 1, 2, 3, and 4 represent the PCR product size of 230 bps for different subjects. Lane no. M represents the 50 bp ladder.

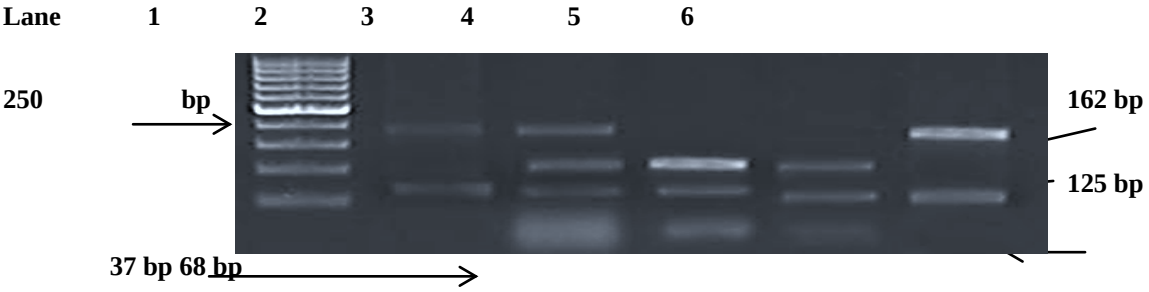

Figure 3: representative gel picture showing the RFLP results for RFC gene. lane no. 1 represents the 50 bp ladder. Lane no. 2 and 6 represent the homozygous AA condition. Lane no. 3 represents the heterozygous GA condition. Lane no. 4 and 5 represent the homozygous GG condition.

Table 2: Frequency of RFC alleles in CRC patients and controls

ALLELOTYPE	Cases	Controls	Odds ratio	95% confidence interval	P value
	n^s (%)	n^s(%)			
G	284 (47.33%)	329 (51.4%)	Referent	--------	-------
A	316 (52.67%)	311 (48.6%)	1.17	0.9418-1.471	0.15

n^s= No. of Alleles; *P using $\chi 2$ test; P<0.05 (Data statistically significant)

From allele typing it was observed that the frequency of RFC G allele was found to be less (47.33%) in cases than in(51.4%) controls.While as reverse was seen for the A allele which showed a frequency of 52.67% in cases and 48.6% in controls.Cases with A allele showed a 1.17 fold risk in comparison to the wild allele G though the result was not statistically significant (P= 0.15).

Table 3: Distribution of RFC 1 genotypes in colorectal cancer cases and controls.

Polymorphism / genotype	Cases n (%)	Controls n (%)	OddsRatio (OR)	95% Confidence Interval(CI)	P value
GG	92 (30.67%)	113 (35.32%)	Referent	-----	------
GA	100 (33.33%)	103 (32.18%)	1.19	0.8080 - 1.760	0.37
AA	108 (36.0%)	104 (32.50%)	1.27	0.8678 - 1.875	0.21

***P using $\chi 2$ test; P<0.05 (Data statistically significant)**

On genotypic distribution, the homozygous GG genotype was found to be present in 92(30.67%) of the cases and 113 (35.32%) of the controls, the heterozygous GA variant was seen in 100 (33.33%) of the cases and 103 (32.18%) of controls, and the AA homozygous genotype was detected in 108 (36.0%) of cases and 104 (32.50%) of controls implying that the frequency of homozygous AA and heterozygousGA variant was more in colorectal cancer patients than GG (wild type) which was more in controls. The most common genotype found in cases was AA and in controls it was GG.

When stratified for several variable characteristics (Table 4), age group distribution revealed that above 50 age group had relatively higher number of the three genotypes (56, 61, and 61 for GG, GA and AA) as compared to below 50 age group(GG=36;GA=39;AA=47). When dwelling was taken as a criteria, rural dwellers showed frequencies of [GG=71(77.18%); GA= 73(73%) and AA= 75(69.45%)] that were higher than the urban dwellers [GG=21(22.82%); GA= 27(27%); AA= 33(30.55%)]. For gender stratification, males showed highest frequency of the AA genotype (64.81%) than GG (58.7%) or GA (41%) while as females had GA (59%) as the most predominant genotype followed by GG (41.3%) and AA (35.19%). Smokers showed a highest frequency of AA genotype (62.97%) as compared to non-smokers which had a higher frequency GG (63.05%). P value= 0.001denoted statistical significance. Subjects with cancer in colonic region showed AA genotype dominance (72.22%) as compared to subjects with rectal cancer which showed higher frequency of GG genotype (57.6%).

To evaluate the significance of RFC polymorphism individually, we further stratified it in colon cancer patients Table 5 shows association of RFC variants among the different categories of colon cancer patients. Female colon cancer patients showed higher frequency of GG (53.85%) and GA (70%) genotypes while as male patients had higher AA genotype (60.26%). Smoking status also showed higher AA genotype frequency (89.74%) for smokers as compared to non-smokers (10.26%).

Study showed that there was a similar distribution of urban and rural dwellers among general colorectal cancer and colon cancer groups. Majority of the patients came from rural areas. When age of patients (cases) was categorized, two general categories ($\leq$50 and >50) were included in the initial analysis to begin with. Subjects above 50 showed higher frequencies of GG, GA and AA genotype as compared to below 50 subjects.

Table 4:Clinicopathological Characteristics of Colorectal Cancer Patients and *RFCA80G* Polymorphism

Variables		Total	GG	GA	AA	χ^2, P value
		n=300 (%)	n=92 (%)	n=100 (%)	n=108 (%)	
Age	≤ 50	122 (40.6%)	36 (39.13%)	39 (39 %)	47 (43.51%)	
	> 50	178 (59.4%)	56 (60.86%)	61 (61%)	61 (58.48%)	0.56, 0.75
Gender	Male	165 (55%)	54 (58.7%)	41 (41%)	70 (64.81%)	**12.63, 0.001**
	Female	135 (45%)	38 (41.3%)	59 (59%)	38 (35.19%)	
Dwelling	Urban	81 (27%)	21 (22.82%)	27 (27%)	33 (30.55%)	1.50, 0.47
	Rural	219 (73%)	71 (77.18%)	73 (73%)	75 (69.45%)	
Smoking	Smoker	153 (51%)	34 (36.95%)	51 (51%)	68 (62.97%)	**13.45, 0.001**
	Nonsmoker	147 (49%)	58 (63.05%)	49 (49%)	40 (37.03%)	
Location	Colon	157(52.33%)	39 (42.4%)	40 (40%)	78 (72.22%)	**26.87,0.0001**
	Rectum	143(47.67%)	53 (57.6%)	60 (60%)	30 (27.78%)	

Table 5: Significance of *RFC*I Polymorphism with Respect to Characteristics of Colon Cancer Patients

Variables		Total	GG	GA	AA	χ^2, P value
		n=157 (%)	n=39 (%)	n=40 (%)	n=78 (%)	
Gender	Male	77 (49%)	18 (46.25%)	12 (30%)	47 (60.26%)	**9.85, 0.007**
	Female	80 (51%)	21(53.85%)	28 (70%)	31 (39.74%)	
Dwelling	Urban	56(35.67%)	12 (30.77%)	15 (37.5%)	21 (26.92%)	1.39, 0.49
	Rural	101(64.33%)	27 (69.23%)	25 (62.5%)	57 (73.08%)	
Smoking	Smoker	120 (76.43%)	22 (56.41%)	28 (70%)	70 (89.74%)	**17.27, 0.0002**
	Nonsmoker	37 (23.57%)	17 (43.59%)	12 (30%)	8 (10.26%)	

Although our study did not show an overall statistically significant association of RFCpolymorphism with the risk of CRC but we found that both the GA heterozygous and the AA homozygous variants showed a 1.19(OR= 1.19, 95%CI, 0.8080- 1.760) and 1.27 (OR=1.27, 95%CI, 0.8678-1.875) fold increased risk as compared to wild homozygous GG genotype. Our study showed statistically significant results when gender, smoking and tumor location characteristics were stratified with RFCpolymorphism. Study on contrary showed no significant association between RFC polymorphism and age or dwelling among colorectal cancer patients in general or colon cancer in specific.

5. Discussion

Because of its important role, folate levels affect both DNA repair and gene expression. Folate deficiency leads to large scale mis incorporation of uracil into DNA and chromosome breaks. This in turn induces chromosome damage, fragile site formation, micronucleus formation and elevated uracil levels in the DNA [35]. Any functional alterations due to polymorphisms in the genes involved in folate transport and metabolism may be associated with the development of cancers [36].

From the critical role of RFC in membrane transport of folates for cellular proliferation and tissue regeneration and of antifolates for cancer therapy, interest in RFC gene variants is particularly acute. Also mean expression levels of the RFC1 protein have been shown to be higher in tumor tissues compared with normal colonic mucosa [37].Therefore, is good candidate for studying the effect of polymorphisms in folate transporting genes on the development of malignancies like colorectal cancer.

Taking this in account, the aim of our study was to assess the RFC polymorphic variants in our population and correlate them with CRC susceptibility. We found out that the G allele was more common in the general population in comparison A allele was more frequent with a 1.17 fold risk in cases than in controls. Genotypic distribution also showed more of homozygous GG and heterozygous GA genotypes in normal population while as homozygous AA genotype showed higher distribution in cases. Though the results were not statistically significant however we observed that individuals with the AA and GA genotype were at a 1.27 fold and a 1.19 fold increased risk in comparison to individuals containing the GG wild genotype. The variant A allele is consistently and linearly associated with higher plasma folate concentrations or in other words lesser cellular uptake [38].

Arg27 is predicted to lie in the first transmembrane domain, a region implicated as important to carrier function [38-42]. This RFC G80A alteration, at position 80 of the coding region results in an amino acid substitution (*i.e.*, Arg/His27). Inactivating mutations in the RFC coding region abolish its transport activity, thereby resulting in altered folate uptake and antifolate resistance [38, 39] and [43]. Similar to our results have been observed by [24, 26, 44,45].Whether the *RFC*1 80G>A variant induces shifts in intracellular folate redistribution is not known. In addition, other regulating factors in cellular folate metabolism might counteract the diminished influx. Thus, patients carrying the *RFC*1 80A allele may have lower folate or methotrexate (MTX) uptake, which might increase cancer risk and decrease MTX efficacy. Interestingly, the *RFC*1 gene is located on chromosome 21. Extra copies of this chromosome in favorable subgroups may be associated with enhanced RFC1 mRNA expression and MTX-PG accumulation [46] and, hence, may be a selective advantage.

The association of RFC polymorphism with gender where males had higher frequency of AA (64.81%; 60.26%) and females with GA (59%; 70%) genotypes respectively in case of colorectal cancer (χ^2 =**12.63, P= 0.001**) and in colon cancer (χ^2=**9.85,P=0.007)** alone points towards complex interactions between gender-related differences in exposure to hormones and risk factors, and how they interact with two different kinds of RFC proteins, also observed byCornelia et al.(2005)[24].

Our study also yielded a positive statistical interaction between the occurrence of colon cancer and RFC polymorphism, which was consistent with the studies carried out by Cornelia et al.(2005) [24].We found statistically significant results for smoking for RFC polymorphism in CRC patients when stratification was done in general for CRC (χ^2=**13.45, P= 0.001)** and for colon cancer alone(χ^2 = **17.27, P= 0.0002**).The reason could be that the large number of individuals of this population are relatively high consumers of tobacco (in the form of Hukka for rural and cigarette for urban areas), apart from other rich sources of carcinogens suggested to have a strong role to play in the etiology of cancers [47-49].Current and former smokers are more likely to develop colorectal cancer than lifelong nonsmokers (defined as smoking fewer than 100 cigarettes in their lives). Similar results to ours were shown by [50] and [51].

CRC is a complex disease, and both environmental and genetic factors are involved in the development of the disease. Our observation thus supports the notion that the effect of single genetic factor (RFC polymorphism in this case) on the risk of CRC may be more pronounced in the presence of other common genetic or environmental risk factors such as smoking.

6. Conclusion

Our study has shown the variants of RFC gene involved in folate transport with respect to CRC patients within a homogenous ethnic group. Our study is the first in our knowledge to investigate prospectively the frequency and relation of the RFC gene polymorphism with colorectal cancer in Kashmiri population. It was observed that RFC AA variant genotype and GA genotype have a 1.27 and 1.19 fold increased risk of CRC respectively. Significant association of RFC polymorphism was observed for gender and location of cancer with CRC susceptibility. RFC polymorphism showed significant association with CRC in smokers.

Due to the historical, cultural, religious and lifestyle differences, Kashmiri's show wide genetic diversities.Also a limitation to this study is a limited sample number. Thus, to confirm our findings and to clarify the contribution of this gene in the development and prognosis of CRC, more exhaustive studies on a large scale need to be carried out on our population.

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References

[1] E. Giovannucci, M. J. Stampfer, G. A. Colditz, D.J. Hunter, C. Fuchs,B. A. Rosneret al."Multivitamin use, folate, and colon cancer in women in the Nurses' Health Study."*Ann Intern Med*. Vol. 129, pp. 517–4,1998

[2] M.A. Sanjoaquin, N. Allen, E. Couto, A. W. Roddam, T. J. Key."Folate intake and colorectal cancer risk: a meta-analytical approach."*Int J Cancer*. Vol. 113, pp. 825–8, 2005

[3] Y.I. Kim. "Folate and colorectal cancer: an evidence-based critical review."*MolNutr Food Res*. Vol. 51, pp. 267–2, 2007

[4] Y.I. Kim. "Folic Acid supplementation and cancer risk: point."*Cancer Epidemiol Biomarkers Prev*. Vol. 17, pp. 2220–5, 2008

[5] C.M. Ulrich. "Folate and cancer prevention-where to next? Counterpoint." *Cancer Epidemiol Biomarkers* Prev. Vol. 17, pp. 2226–0, 2008

[6] L. C. Bollheimer, R. Buettner, A. Kullmann, F. Kullmann."Folate and its preventive potential in colorectal carcinogenesis. How strong is the biological and epidemiological evidence?"*Crit Rev OncolHematol*. Vol. 55, pp.13–6, 2005

[7] L. H. Matherly, Z. Hou, Y. Deng. "Human reduced folate carrier: translation of basic biology to cancer etiology and therapy. "*Cancer Metastasis Rev*. vol. 26, pp. 111–8, 2007

[8] R. Rosin-Arbesfeld, F. Townsley, M. Bienz."The APC tumour suppressor has a nuclear export function."*Nature.*Vol. 406(6799): pp. 1009-2, 2000

[9] F. M. Sirotnak and B. Tolner. "Carriermediated membrane transport of folates in mammalian cells."*Annu Rev Nutr*. Vol. 19,pp. 91-2, 1999

[10] R. Zhao, L.H. Matherly, and I.D. Goldman. "Membrane transporters and folate homeostasis: intestinal absorption and transport into systemic compartments and tissues."*Expert Rev Mol Med*. 11: e4 2009

[11] I. Ifergan, G. Jansen, Y. G. Assaraf. "The reduced folate carrier (RFC) is cytotoxic to cells under conditions of severe folate deprivation. RFC as a double edged sword in folate homeostasis."*J Biol Chem*. Vol. 283, pp. 20687–5, 2008

[12] K. E. Brigle, M. J. Spinella, E. E. Sierra, I. D. Goldman."Characterization of a mutation in the reduced folate carrier in a transport defective L1210 murine leukemia cell line."*J Biol. Chem*. Vol. 270, pp. 22974 –9, 1995

[13] H. Sadlish, R. C. Murray, F. M. Williams, W. F.Flintoff. "Mutations in the reduced-folate carrier affect protein localization and stability."Biochem. J. vol. 346,pp. 509–8, 2000

[14] L. H. Matherly and I. D. Goldman. "Membrane transport of folates."*VitamHorm. Vol.* 66, pp.403–6, 2003

[15] R. Zhao and I. D. Goldman. " Resistance to antifolates."*Oncogene* .vol. 22, pp**.** 7431–7, 2003

[16] I. D. Goldman, L. H. Matherly. "The cellular pharmacology of methotrexate."*PharmacolTher*. Vol. 28(1), pp. 77–2

1985

[17] A. A. Adjei, O.E. Salavaggione, S.J. Mandrekar,G.K. Dy, K.L. Ziegler, C. Endo, et al. "Correlation between polymorphisms of the reduced folate carrier gene (*SLC19A1*) and survival after pemetrexed-based therapy in non-small cell lung cancer."*J ThoracOncol.* Vol. 5, pp. 1346–3, 2010

[18] A. Chango, N. Emery-Fillon, C. P. de Courcy, D. Lambert, M. Pfister, D. S. Rosen- blatt et al. "A polymorphism (80G->A) in the reduced folate carrier gene and its associations with folate status and homocysteinemia."*Molecular Genetics and Metabolism.* Vol. 70(4), pp. 310-5, 2000

[19] J.R. Whetstine, A.J. Gifford, T. Witt, X.Y. Liu, R. M. Flatley, M. Norris et al. "Single nucleotide polymorphisms in the human reduced folate carrier: characterization of a high-frequency G/A variant at position 80 and transport properties of the His (27) and Arg (27) carriers". *Clinical Cancer Research.*Vol. 7(11), pp. 3416-2, 2001

[20] L.H. Matherly. "Human reduced folate carrier gene and transcript variants: functional, physiologic, and pharmacologic consequences". *Current Pharmacogenetics*. Vol. 2, pp. 287–8, 004

[21] L. Sharp and J. Little. "Polymorphisms in genes involved in folate metabolism and colorectal neoplasia: a HuGE review". *Am J Epidemiol*. Vol. 159, pp. 423–3, 2004

[22] J. Ma, M. J. Stampfer, E. Giovannucci, C. Artigas, D.J. Hunter, C.Fuchs, et al. "Methylenetetrahydrofolatereductase polymorphism, dietary interactions, and risk of colorectal cancer". *Cancer Res*. Vol. 57, pp. 1098–2, 1997

[23] C. F. Skibola, M. S. Forrest, F. Coppede, L. Agana, A. Hubbard, M. T. Smith, et al. "Polymorphisms and haplotypes in folate-metabolizing genes and risk of non-Hodgkin lymphoma."*Blood.* Vol. 104, pp. 2155–2, 2004

[24] Cornelia M. Ulrich, Karen Curtin, John D. Potter, Jeannette Bigler, Bette Caan, L. Martha and Slattery. "Polymorphisms in the reduced folate carrier, thymidylate synthase, or methionine synthase and risk of colon cancer."*Cancer Epidemiol Biomarkers Prev.*vol. 14, pp. 2509–6, 2005

[25] Anita Koushik, Peter Kraft, Charles S. Fuchs, Susan E. Hankinson, Walter C. Willett, Edward L. Giovannucciet al. "Non synonymous polymorphisms in genes in the one-carbon metabolism pathway and associations with colorectal cancer."*Cancer Epidemiol Biomarkers Prev.* vol. 15,pp. 2408–7, 2006

[26] L. Wang, W. Chen, J. Wang, Y. Tan, Y. Zhou, W. Ding,et al. "Reduced folate carrier gene G80A polymorphism is associated with an increased risk of gastro esophageal cancers in a Chinese population."*Eur J Cancer*. Vol. 42, pp. 3206–1, 2006

[27] V. Eklof, B. Van Guelpen, J. Hultdin, I. Johansson, G. Hallmans, R. Stenling, et al. "The reduced folate carrier (RFC1) 80G>A and folate hydrolase 1 (FOLH1) 1561C>T polymorphisms and the risk of colorectal cancer: a nested case-referent study". *Scand J Clin Lab Invest*. pp. 1–9, 2007

[28] A. Hazra, K. Wu, P. Kraft, C. S. Fuchs, E. L. Giovannucci, D. J. Hunter, et al. "Twenty-four non-synonymous polymorphisms in the one-carbon metabolic pathway and risk of colorectal adenoma in the Nurses' Health Study."*Carcinogenesis.* Vol. 28, pp. 1510–9, 2007

[29] T. Gotze, C. Rocken, F.W. Rohl, T. Wex, J. Hoffmann, S. Westphal, et al. "Gene polymorphisms of folate metabolizing enzymes and the risk of gastric cancer". *Cancer Lett* .vol. 251, pp. 228–6, 2007

[30] M. M. Mir, N. A. Dar, S. Gochhait, S. A. Zargar, A. G. Ahangar, R. N. Bamezai. "p53 mutation profile of squamous cell carcinomas of the esophagus in Kashmir (India): a high-incidence area."*Int. J. Cancer*.vol. 116, pp. 62-8, 2005

[31] I. Murtaza, D. Mushtaq, M. A. Margoob, A. Dutt, N .A. Wani, I. Ahmad, et al. "A study on p53 gene alterations in esophageal squamous cell carcinoma and their correlation to common dietary risk factors among population of the Kashmir valley". *World J Gastroenterol*, vol. **12**, pp. 4033-7, 2006

[32] A. S. Sameer, S.Rehman,.Arshad A. Pandith, NiddaSyeed, Zaffar A. Shah, Nissar A. Chowdri*et al*. "Molecular gate keepers succumb to gene aberrations in colorectal cancer in Kashmiri population, revealing a high incidence area".*Saudi J Gastroenterol*. Vol. 15,pp. 244-2, 2009

[33] A. S. Sameer, Z. A. Shah, N. Syeed, M. Z. Banday, S. M. Bashir, B. A.Bhat*et al*. "TP53 Pro47Ser and Arg72Pro polymorphisms and colorectal cancer predisposition in an ethnic Kashmiri population". *Genet. Mol. Res*. Vol. 9, pp. 651-0, 2010

[34] M. Siddiqi, R. Kumar, Z. Fazili, B. Spiegelhalder, R. Preussmann. "Increased exposureto dietary amines and nitrate in a population at high riskof oesophageal and gastric cancer in Kashmir (India)".*Carcinogenesis*, vol. 13, pp. 1331-5, 1992

[35] A. Hishida, K. Matsuo, N. Hamajima, H Ito, M Ogura, Y Kagami, et al. "Associations between polymorphisms in the thymidylate synthase and serine hydroxymethyltransferase genes and susceptibility to malignant lymphoma". *Haematologica*.Vol. 88, pp.159-6, 2003

[36] Y.I. Kim."Folate and carcinogenesis: evidence, mechanisms, and implications". *J NutrBiochem.* Vol. 10, pp. 66-8, 1998

[37] E. Odin, Y. Wettergren, S. Nilsson, R. Willen, G. Carlsson, C. P. Spears, et al. "Altered gene expression of folate enzymes in adjacent mucosa is associated with outcome of colorectal cancer patients". *Clin Cancer Res*. Vol.9: pp. 6012–9, 2003

[38] S. Drori, G. Jansen, R. Mauritz, G. Peters, Y. G. Assaraf. "Clustering of mutations in the firsttransmembrane domain of the human reduced folate carrier in GW1843U89-resistant leukemia cells with impaired antifolate transport and augmented folate uptake". *J Biol Chem. vol.* 275, 30855–6, 2000.

[39] G. Jansen, R. Mauritz, S. Drori, H. Sprecher, I. Kathmann, M. Bunni, et al. "A structurally altered human reduced folate arrier with increased folic acid transport mediates a novel mechanism of antifolate resistance". *J Biol Chem*.

vol. 273, pp.30189–98, 1998

[40] R. Zhao, Y. G. Assaraf, I. D. Goldman. "A mutated reduced folate carrier (*RFC1*) with increased affinity for folic acid, decreased affinity for methotrexate, and an obligatory anion requirement for transport function". *J. Biol. Chem*. Vol. *273*, pp.19065–71, 1998

[41] R. Zhao, Y. G. Assaraf, I. D. Goldman."A reduced folate carrier mutation produces substrate-dependent alterations in carrier mobility in murine leukemia cells and methotrexate resistance with conservation of growth in 5-formyltetrahydrofolate". *J. Biol. Chem.*vol. *273*, pp.7873–79, 1998

[42] A. Tse, K. Brigle, S. M. Taylor, R. G. Moran. "Mutations in the reduced folate carrier gene which confer dominant resistance to 5,10-dideazatetrahydrofolate". *J. Biol. Chem*. Vol. 273, pp. 25953–60, 1998

[43] L. Rothem, I. Ifergan, Y. Kaufman, D. G. Priest, G. Jansen, Y. G. Assaraf. "Resistance to multiple novel antifolates is mediated via defective drug transport resulting from clustered mutations in the reduced folate carrier gene in human leukaemia cell lines". *Biochem J Vol.*367, pp. 741–50, 2002

[44] J. Lissowska, M. M. Gaudet, L. A. Brinton, S. J. Chanock, B. Peplonska. "Genetic polymorphisms in the one-carbon metabolism pathway and breast cancer risk: a population-based case-control study and meta-analyses". *Int J Cancer*. vol. 120, pp. 2696–3, 2007

[45] L. E. Moore, N. Malats, N. Rothman,F. X. Real, M. Kogevinas, S. Karami, et al. "Polymorphisms in one-carbon metabolism and trans-sulfuration pathway genes and susceptibility to bladder cancer". *Int J Cancer*. Vol. 120, pp. 2452–8, 2007

[46] V. M. Belkov, E. Y. Krynetski, J. D. Schuetz, Y. Yanishevski, E. Masson, S. Mathew, et al."Reduced folate carrier expression in acute lymphoblastic leukemia: a mechanism for ploidy but not lineage differences in methotrexate accumulation". *Blood.* vol. 93, pp.1643–50, 1998

[47] A. K. Chakravarti. "Regional preferences for food: some aspects of food patterns in India". *Can Geogr*, vol. 18, pp. 395-10, 1974

[48] M. Siddiqi, A. R. Tricker, R. Preussmann."The occurrence of preformed N-nitroso compounds in the food samples from a high risk area of esophageal cancer in Kashmir, India". *Cancer Lett*, vol. 39, pp. 37-43, 1988

[49] N. A. Dar, G .A. Bhat, I A. Shah, B. Iqbal, M. A.Makhdoomi, I.Nisar, et al. "Hookah smoking, nass chewing and oesophageal cell carcinoma in Kashmir, India". *Br J Cancer*, vol. 107, pp. 1618-23, 2012

[50] L. Le Marchand, L. R. Wilkens, L. N. Kolonel, J. H. Hankin, L. C. Lyu. "Associations of lifestyle, obesity, smoking, alcohol use, and diabeteswith the risk of colorectal cancer". *Cancer Res.* vol. 57, pp. 4787–94, 1997

[51] M. L. Slattery, J. D. Potter, G. D. Friedman, K. N. Ma, S. Edwards. "Tobacco use and colon cancer". *Int. J. Cancer*, vol. *70,pp.* 259–4, 1997.